Characterization and Reclassification of Yeasts Used for Biological Control of Postharvest Diseases of Fruits and Vegetables

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In previous studies workers have shown that three yeast strains (strains US-7, 82, and 101) have biological control activity against various postharvest fungal pathogens of fruits and vegetables, including *Penicillium* rots of apples and citrus and *Botrytis* rot of apples. In these reports the researchers have described these strains as *Debaryomyces hansenii* (anamorph, *Candida famata*) or *Candida* sp. strains. In this study we performed additional physiological, DNA reassociation, and mannan characterization tests that clearly established a new taxonomic classification for these strains, *Candida guilliermondii*. We also propose amendment of the physiological test profile in the taxonomic description of *C. guilliermondii*.

Postharvest fungal decays of fresh fruits and vegetables can result in serious losses (1). Traditionally, these losses have been controlled by applications of fungicides after harvest or prior to shipping to markets. Alternative methods for control of these losses have been investigated because fungicides are being removed from the market due to human health risk concerns (4). In addition, fungicides frequently become ineffective due to their tendency to select for fungicide-tolerant strains of the pathogens (3, 15).

Postharvest treatment of fruits with microorganisms recovered from fruit surfaces has shown promise as an alternative method for the control of postharvest diseases of apples, citrus, and other fruits (21). In several reports workers have shown that certain yeast strains are effective for biological control of postharvest fungal decays of apples (13) (grey and blue molds, caused by *Botrytis cinerea* Pers. ex Fr. and *Penicillium expansum* Link ex Thom., respectively) and citrus (5, 6, 20) (blue mold, caused by *Penicillium digitatum* Sacc. or *Penicillium italicum* Wehmer). These yeasts do not exhibit antibiosis toward the fungal decay pathogens in vitro and have been variously identified as *Candida* sp. Berkhout (13) or *Debaryomyces hansenii* (Zopf) Lodder et Kreger-van Rij (anamorph, *Candida famata* ((Harrison) Meyer et Yarrow)).

The studies in which the taxon *Debaryomyces hansenii* was used (5, 6, 20) were based on morphological and phenotypic characterizations that were determined initially by workers at the American Type Culture Collection, Rockville, Md. A second characterization by workers at the American Type Culture Collection indicated that the strains were a *Candida* sp. having uncertain affiliation to known species. These yeast strains (strains US-7, 82, and 101) do not exhibit a teleomorphic state in culture.

The criteria used for classification of these yeast strains are based primarily on the work of Kreger-van Rij (9), who provided morphological, physiological, and biochemical data for the differentiation of yeast strains. Differentiation at the species level is based on the results of a number of carbon utilization and fermentation tests. However, the number of tests available for differentiation in this system is apparently inadequate in some circumstances. More stringent means of taxonomic differentiation include (i) comparison of cell wall mannans by determining proton nuclear magnetic resonance (NMR) spectra (8) and (ii) determination of percentages of homology of genomic DNAs by DNA-DNA hybridization (11).

Because of the inadequacy of standard criteria for differentiation of the yeast strains that are effective biological control agents, further research was needed to determine the taxonomic affinity of these organisms. The resulting data may also prove to be useful in the utilization and manipulation of these strains to facilitate improved biological control of postharvest fruit diseases. Our evaluations were performed by using an extended set of criteria for taxonomic differentiation, including standard biochemical utilization and fermentation tests, characterization of cell wall mannans by proton magnetic resonance spectroscopy, and determination of levels of genomic homology with type specimens.

MATERIALS AND METHODS

Strains and culture methods. Three yeast strains that are effective biological control agents, strains 82 (= NRRL Y-18313), US-7 (= NRRL Y-18314), and 101 (= NRRL Y-17257), were isolated from the surfaces of lemon fruits as previously described (20). Cultures were routinely stored on silica gel at −10°C and were recovered by plating onto nutrient dextrose agar (0.8% nutrient broth, 0.5% yeast extract, 1.0% dextrose, 1.5% agar, pH 7.0) and subsequent incubation at 28°C for 24 to 48 h. Type strains of the yeast species *D. hansenii* (strain NRRL Y-7426) and *Pichia guilliermondii* Wickerham (anamorph, *Candida guilliermondii* (Castelani) Langeron et Guerra) (strain NRRL Y-2075) were used for comparison purposes and are maintained in the

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Physiological and morphological characterization. The methods which we used for evaluating morphology and for performing the fermentation and assimilation tests have been described previously (9, 19). Physiological tests were repeated at least once for each of the test strains. The type strains of *P. guilliermondii* and *D. hansenii* were included in physiological tests that were critical for the classification of the strains, including the tests for assimilation of lactose, melibiose, DL-lactate, and soluble starch, fermentation (all sugars), growth in the absence of vitamins, and growth in the presence of 50% glucose and 10% NaCl.

Isolation and characterization of mannans. Starter cultures of yeast cells were prepared by inoculating 10 ml of yeast extract-malt extract broth (3 g of yeast extract, 5 g of Bacto-Peptone [Difco Laboratories], 3 g of malt extract, 10 g of dextrose [Difco], pH 6.2 prior to sterilization) (19) with a partial loopful of cells from an overnight nutrient dextrose agar culture. Starter cultures were shaken overnight at 300 rpm on a rotary shaker at room temperature. For each strain, 10 ml of culture was used to inoculate 1 liter of yeast extract-malt extract broth contained in a 2.8-liter Fernbach flask. The flasks were shaken for 2 days at 250 rpm at room temperature. The cells were sedimented from the turbid cultures by centrifugation at 16,300 × *g* for 20 min. The supernatant was poured off, the pelleted cells were washed once with distilled water, and the cells were collected by centrifugation, suspended in distilled water, and then lyophilized.

Mannans were isolated by using the procedure of Gorin and Spencer (8). The neutral sugar compositions of the samples were determined by gas-liquid chromatography as previously described (7). ^{1}H-NMR spectra were obtained in D_2O by using dimethyl sulfoxide (2.5 ppm) as the internal standard, a JOEL model CX400 spectrometer, and the following parameters: probe temperature, 70°C; acquisition time, 1.6 s; pulse delay, 5.0 s; pulse width, 7.3 μs. Typically, 50 scans were accumulated for each spectrum.

DNA extraction and characterization. DNAs were extracted and purified by using a combination of the procedures of Marmur (12) and Bernardi et al. (2), as described by Price et al. (14). The hyperchromicity values for the purified preparations, as percentages of initial A_{260}, ranged from 36 to 37%.

In preparation for reassociation experiments, DNAs were mechanically sheared in a French press to fragment sizes of ca. 400 nucleotides. The extent of DNA reassociation was determined spectrophotometrically (11, 16, 17) by using a Gilford model 250 recording spectrophotometer and a model 2527 thermoprogrammer. The reaction mixtures contained 75 μg of sheared, denatured DNA per ml in 5× SSC–20% dimethyl sulfoxide (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate). The temperature used for reassociations was 57°C (melting temperature of *P. guilliermondii* DNA minus 25°C).

RESULTS

Morphological and physiological characteristics. The gross morphological characteristics of the strains are described below. After 1 month on glucose-yeast extract-peptone agar at 25°C, the colonies of each of the strains were white, soft, glistening, and smooth. On solid yeast extract-malt extract agar the cells were unicellular. After 1 day in yeast extract-malt extract broth, small globose cells were observed mainly in chains or clusters; many cells had one bud. Reactions with diazonium blue B, which positively stains cell walls of basidiomycetous yeasts but not ascomycetous yeasts, were negative for all strains; however, no ascospores were produced in the cultures. Strains US-7 and 101 formed primitive pseudohyphae in Dalmau plate cultures on corn meal agar, whereas strain 82 formed well-developed pseudohyphae. Blastoconidia were not observed in cultures of any of the strains.

Physiological test results are shown in Table 1. All of the yeast biological control strains utilized a broad array of carbohydrates and organic acids. We observed variation in the ability to assimilate certain compounds between strains. Strain 82 did not assimilate L-rhamnose or melibiose, and strain 101 did not assimilate DL-lactate. None of the strains assimilated soluble starch, and all grew in the absence of vitamins.

Characterization of the mannans. The yields of purified mannans from the biological control strains were as follows: strain US-7, 12%; strain 82, 8.5%; and strain 101, 8.6%. The yields for *D. hansenii* NRRL Y-7426 and *P. guilliermondii* NRRL Y-2075 were 7.0 and 5.5%, respectively. Gas-liquid chromatography analysis of the purified mannans showed that mannose was the only neutral sugar present in the preparations. The ^{1}H-NMR spectra of the anomeric region of the mannans from the three biological control strains were identical to each other (data not shown) and to the spectrum for the mannan of *P. guilliermondii*, but differed from the spectrum for the mannan of *D. hansenii* (Fig. 1).

DNA reassociation study. The results of the DNA reassociation tests are shown in Table 2. DNA from each of the strains showed a high degree of homology with DNA from the type strain of *P. guilliermondii*; the reassociation values ranged from 94 to 99%. The reassociation values with DNA from the type strain of *D. hansenii* were 14% or less.

DISCUSSION

The biological control yeast strains are taxonomically similar to *C. guilliermondii* based on morphology, mannan data, and the results of DNA reassociation tests. All of the biological control strains differ from *C. famata* in each of these parameters. The results of our physiological characterizations varied from the results of the previously reported phenotypic profile of *C. guilliermondii* (9), demonstrating that physiological characterization alone is inadequate for identification of these strains.

The physiological tests revealed minor differences among the biological control yeast strains in their ability to assimilate L-rhamnose, melibiose, and DL-lactate. These characterizations also showed that yeast strains US-7, 101, and 82 differed from *C. guilliermondii* and *C. famata* in a few tests. In their inability to ferment raffinose these organisms differed from the type strain of *C. guilliermondii*. In addition, all of the strains grew in the absence of vitamins; according to Kreger-van Rij (9), *C. guilliermondii* and *C. famata* do not grow under these conditions. Most importantly, our results indicated that modification of the phenotypic description of *C. guilliermondii* (9) is needed.

Morphological characterization demonstrated that none of the strains formed a sexual state (*Pichia guilliermondii*) in culture. The negative diazonium blue B test indicated that these organisms are ascomycetous yeasts. The ability of all of the strains to form pseudomycelia is consistent with the description of *C. guilliermondii*. Strains of *C. famata* may produce short branched chains of cells instead of pseudomycelia.

TABLE 1. Comparison of fermentation and assimilation test results for the biological control yeast strains with previously published results for *C. guilliermondii* and *C. famata*[a]

Test	Strain 82	Strain US-7	Strain 101	*C. guilliermondii*	*C. famata*
Fermentation of:					
D-Glucose	+[b]	+	+	+ (+)[c]	v (+)
D-Galactose	−	−	−	v (+)	− (+)
Maltose	−	−	−	− (−)	− (−)
Sucrose	+	+	+	+ (+)	v (+)
Lactose	−	−	−	− (−)	− (−)
Raffinose	−	−	−	+/w (w)	v (w)
Assimilation of:					
D-Glucose	+	+	+	+ (+)	+ (+)
D-Galactose	+	+	+	+	+
L-Sorbose	+	+	+	+/s	v
D-Glucosamine	+	+	+	+[d]	NL
D-Ribose	+	+	+	v	v
D-Xylose	+	+	+	+	+
L-Arabinose	+	+	+	+/s	+
D-Arabinose	+	+	+	+	v
L-Rhamnose	−	+	+	v	v
Sucrose	+	+	+	+	+
Maltose	+	+	+	+	+
Trehalose	+	+	+	+	+
Methyl-α-D-glucoside	+	+	+	+[d]	NL
Cellobiose	+	+	+	+	+
Salicin	+	+	+	+	+
Melibiose	−	+	+	+ (+)	v (+)
Lactose	−	−	−	− (−)	v (−)
Raffinose	+	+	+	+	+
Melezitose	+	+	+	+	+
Inulin	+	+	+	+[d]	v
Soluble starch	−	−	−	− (−)	v (+)
Glycerol	+	+	+	+	+
Erythritol	−	−	−	− (−)	v (−)
Ribitol	+	+	+	+	+
D-Glucitol	+	+	+	+	+
D-Mannitol	+	+	+	+	+
Galactitol	+	+	+	+/s	v
myo-Inositol	−	−	−	−	−
2-Keto-D-gluconate	+	+	+	NL	NL
5-Keto-D-gluconate	−	−	−	NL	NL
D-Gluconate	+	+	+	v[d]	NL
DL-Lactate	+	+	−	v (+)	+ (−)
Succinate	+	+	+	v[d]	+
Citrate	+	+	+	v	v
Methanol	−	−	−	NL	NL
Ethanol	+	+	+	NL	NL
Nitrate reduction	−	−	−	−	−
Growth without vitamins	+	+	w	− (−)	v (−)
Growth at 37°C	+	+	+	+ (+)	v (+)
Growth in the presence of:					
50% D-glucose	+	+	+	NL (+)	+[d] (+)
10% NaCl	+	+	+	+[d] (+)	NL (+)
Starch formation	−	−	−	NL	NL
Diazonium blue B reaction	−	−	−	−	−

[a] Data for assimilation tests for *C. guilliermondii* and *C. famata* are the data listed by Kreger-van Rij (9), unless otherwise noted. The type strains of *P. guilliermondii* (strain NRRL Y-2075) and *C. famata* (strain NRRL Y-7426) were included in the tests for fermentation (all sugars), assimilation of lactose, melibiose, DL-lactate, and soluble starch, growth in the absence of vitamins, and growth in the presence of 50% glucose and 10% NaCl.

[b] +, positive reaction; w, weak reaction; −, negative reaction; v, variable reaction; s, slow reaction; NL, test results not listed by Kreger-van Rij (9).

[c] The data in parentheses are experimental data for the type strains.

[d] Test result listed under *C. guilliermondii* or *C. famata* by Kreger-van Rij (9).

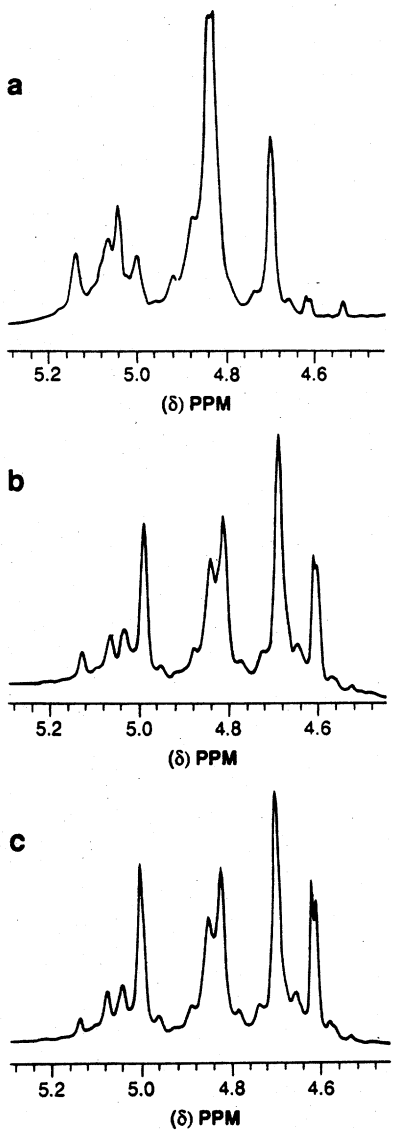

FIG. 1. Comparison of the anomeric regions of the ^{1}H-NMR spectra of yeast cell wall mannans isolated from *D. hansenii* NRRL Y-7426 (a), *P. guilliermondii* NRRL Y-2075 (b), and biological control strain US-7 (c).

The proton NMR spectra of the mannans from the three biological control yeast strains strongly indicate that these strains are not *D. hansenii* strains, but rather belong to group 15a as defined by Gorin and Spencer (8). In addition to *P. guilliermondii*, this group also contains the species *Wingea robertisii* van der Walt, *Debaryomyces vanriji* Abadie, Pignal et Jacob, and *Schwanniomyces occidentalis* Klöcker. As our spectra are much more detailed than those obtained by Gorin and Spencer (8, 18), due to improvements made in NMR instrumentation the past few years, it may now be possible to further distinguish among species included in group 15a.

The DNA reassociation data support identification of these yeasts as members of the species *C. guilliermondii*. Ordinarily, strains that exhibit more than 70% DNA relatedness belong to the same species (10). Unrelated strains have background relatedness values of approximately 0 to 20%.

Strains of *C. guilliermondii* are widely distributed in

TABLE 2. Percentages of reassociation of nuclear DNAs of biological control strains with DNAs from the type strains of *P. guilliermondii* and *D. hansenii*.

Type strain	% Reassociation[a]		
	Strain US-7	Strain 82	Strain 101
P. guilliermondii NRRL Y-2075	99	97	94
D. hansenii NRRL Y-7426	0	0	14

[a] Standard deviation, ≤5%.

nature, having been isolated from various plant and animal sources (6). The type strain of this species was isolated from American elm (*Ulmus americana* L.). The wide distribution of these strains in nature may be due, in part, to their ability to utilize a broad range of substances for growth and to grow under a wide range of temperatures and osmotic conditions. Results from our physiological characterizations show that the biological control strains differ from the type strain and previously described strains of *C. guilliermondii*, suggesting that ecological specialization may occur within the species.

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